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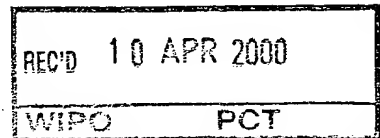
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בקשה לפטנט
Application For Patent

אני, (שם המבקש, מעמו ולגבי גוף מאגדת מקום התאגדותו)
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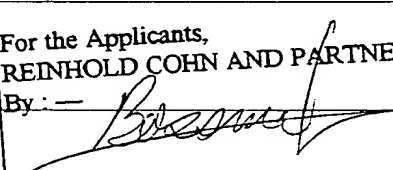
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Induction of nerve generation

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Induction of nerve generation

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The Inventors:
Meir SHINITZKY

הממציאים:
מאיר שיניצקי

C. 112305

NERVE GENERATION

FIELD OF THE INVENTION

The present invention concerns cyclic glycerophosphates and analogs thereof.

5 PRIOR ART

The following is a list of references which is intended for a better understanding of the background of the present invention.

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Linn, L.F.H., *et al.*, *Science*, 260:1130-1134 (1993).

5 Markham, R. and Smith, J.D., *Biochem. J.*, 52:552- (1952).

Shinitzky, M., Friedman, P. and Haimovitz, R., Formation of 1,3-cyclic glycerophosphate by the action of phospholipase C on phosphatidylglycerol, *J. Biol. Chem.*, 268:14109-14115 (1993).

10 Su, B., Kappler, F., Szwergold, B.S. and Brown, T.R., *Cancer Res.*, 53:1751-1754, (1993).

Tomac, A., *et al.*, *Nature*, 373:335-339 (1995).

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BACKGROUND OF THE INVENTION

20 L- α -glycerophosphate (α GP), a key constituent in phospholipid metabolism (Kennedy and Weiss, 1956), is abundant in most biological tissues (Dawson, 1958). β -Glycerophosphate (β GP) is a product of enzymatic (Ukita *et al.*, 1955) and alkaline (Clarke and Dawson, 1976) hydrolysis of phospholipids and is formed through the cyclic phosphodiester intermediate
25 1,2-cyclic glycerophosphate (1,2 cGP) (Ukita *et al.*, 1955; Clarke and Dawson, 1976). 1,2 cGP has been detected in algae species (Boyd *et al.*, 1987) as well as in human cancer tissues (Su *et al.*, 1993). Similarly, α GP can in principle adopt the cyclic form 1,3-cyclic glycerophosphate (1,3 cGP). This compound has
been shown to be formed as an intermediate in the phospholipase C hydrolysis
30 of phosphatidyl glycerol (PG) (Shinitzky *et al.*, 1993) and upon further hydrolysis is converted to α GP.

A six-membered cyclic phosphate of foremost biological importance is cyclic AMP. The ring of cyclic AMP is actually a derivative of 1,3 cGP backbone. Other cyclic phosphates which were detected in biological systems
35 include glucose cyclic phosphodiester (Leloir, 1951), 2',3'-cyclic

phosphodiester (Markham and Smith, 1952), riboflavin-4',5'-cyclic phosphodiester (Forrest and Todd, 1950), myoinositol-1,2-cyclic phosphodiester (Dawson *et al.*, 1971) and cyclic lysophosphatidic acid (Friedman *et al.*, 1996).

5 Except for cyclic AMP and cyclic GMP which have been extensively studied, no specific biological activities have been so far assigned to the other biological cyclic phosphates.

There are several kinds of disorders and diseases which result from deterioration of areas of the brain and loss of neurons. One example of such
10 diseases are neurodegenerative diseases such as Parkinson's disease (PD). Such diseases often involve degeneration of dopamine-producing neurons. Current therapeutic methods are mostly aimed at continuous stimulation of dopamine receptors which, although initially providing symptomatic relief, these drugs gradually lose effectiveness. Furthermore, such drugs do not prevent the
15 progressive degeneration of dopaminergic neurons characteristics of such diseases.

A large number of growth factors such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor, brain derived growth factor and glial derived
20 neurotrophic factor (Knusel B., *et al.*, 1990; Knusel *et al.*, 1991; Linn *et al.*, 1993) stimulate dopaminergic neuron survival and differentiation *in vitro*. In animal models involving induction of Parkinson's disease, the induced animals show improved behavior and an increase in tyrosine hydroxylase (TH), the key enzyme in the dopamine production pathway immunoreactviity when treated with
25 factors like GDNF (Tomic, A. *et al.* 1995) and ciliary neurotrophic factor (CNTF) (Hagg, T. and Varon 1993).

List of compounds and their abbreviations

The following compounds which formulas are presented in Appendix A just before the claims, will be represented herein in the specification by their abbreviations as follows:

- 5 1. 1,3 cyclic glycerophosphate - 1,3 cGP
2. 1,2 cyclic glycerophosphate - 1,2 cGP
3. 3-acyl 1,2 cyclic glycerophosphate (cyclic lysophosphatidic acid) -
c-lysoPA
4. Phenyl 1,3 cGP - P-1,3 cGP
- 10 5. Phenyl 1,2 cGP - P-1,2 cGP
6. 1,3 cyclic propanediol phosphate - 1,3 cPP
7. 1,2 cyclic propanediol phosphate - 1,2 cPP
8. Phenyl 1,3 cPP - P-1,3 cPP
9. Phenyl 1,2, cyclic propanediol phosphate - P-1,2, cPP
- 15 10. Cyclic dihydroxyacetone phosphate - cDHAP
11. Phenyl cyclic dihydroxyacetone phosphate - P-cDHAP

GLOSSARY

20 The following is an explanation of some terms used above and in the following description and claims:

Promoting neural cell differentiation - this term relates to the capability of cyclic phosphates of the invention to cause cells to mature into neural cells after contact therewith. Such activity may be assessed by one of many *in vitro* and *in vivo* assays such as those described in the examples below. An example for an

25 *in vitro* assay would be to grow cells capable of differentiating into nerve cells (e.g. PC12 cells) in the presence of a tested compound and to determine nerve outgrowth in the cells by microscopic evaluation. *In vivo* assays may, for example, involve treatment of animals with injured dopaminergic neurons with

the tested compounds and testing of motional and limb tremor parameters as well as *in situ* determination of molecules associated with dopaminergic transmission in the treated animals.

5 **Target cells** – any cells which have the potential to mature into neural cells. Non-limiting examples of such cells are PC12 and primary brain cells.

10 **Analog** - the term "*analog*" in accordance with the invention relates to any compound including, for example, deoxy analogs and phenyl esters of the cyclic phosphates, preferably, deoxy analogs. The analogs of the cyclic phosphates of the invention are such which are capable of substantially maintaining the activity of the cyclic phosphate from which they originated.

15 **Substantially maintaining** - this term relates to the capability of analogs of the compound of the invention to promote the activity carried out by the compound from which they were derived. The analog's activity will be considered to be substantially maintained wherein the activity is 30% or above, preferably 50% or above, more preferably 70% or above, and most preferably 90% or above the level of the activity of the compound.

20 **Effective amount** – wherein the method of the invention is intended for prevention of a non-desired condition, the term "*effective amount*" should then be understood as meaning an amount of the active compound which, when administered, results in the prevention of the appearance of the said condition.

25 Prevention of such a condition, e.g. a neurodegenerative condition, may be required prior to the appearance of any symptoms of a disease, e.g. in individuals having a high disposition of developing the disease, or when the compositions are used for the treatment of nerve rescue which is expected after nerve injury. Wherein the compositions or methods are intended for treatment of an ongoing

non-desired condition, the term "*effective amount*" should then be understood as meaning an amount of the active compound which is effective in ameliorating or preventing the enhancement of the treated condition and related symptoms. In accordance with the "*in vitro*" aspect, the term "*effective amount*" relates to an amount of the active material which, upon contact with the target cells, causes the differentiation in said cells.

Neural promoting activity – this term encompasses a variety of neural related activities which may be promoted in target cells upon their contact with GCs of the invention. Such activities include but are not limited to promotion of nerve growth, provision of dopaminotrophic supporting environment in a diseased brain, prevention of nerve degeneration, and nerve rescue.

Prevention or treatment – the term prevention of disorders and diseases is to be understood in accordance with the invention as a reduction in the probability of the appearance of such disorders and diseases in an individual having a high predisposition of developing such disorders and diseases, reducing the extent of the symptoms associated with such disorders and diseases when they occur or completely preventing their appearance.

Treatment of such disorders or diseases in accordance with the invention means ameliorating the symptoms associated with the disorders or diseases, reducing the extent of such symptoms or completely eliminating them.

SUMMARY OF THE INVENTION

In accordance with the invention it has surprisingly been found that 1,2 cGP, 1,3 cGP and some of their analogs are capable of promoting neuronal outgrowth of PC12 adrenal tumorigenic cells in culture after a short incubation period.



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As used herein the term "*alkyl*" refers to an alkyl group having from about 1 to about 24 carbon atoms, e.g. preferably from about 3 carbon atoms to about 20 carbon atoms, most preferably from about 5 carbon atoms to about 15 carbon atoms; the term "*acyl*" refers to an aliphatic saturated or unsaturated C₁ - C₂₄ acyl group, preferably an acyl group having an even number of carbon atoms, most preferably an acyl group derived from a natural fatty acid such as a saturated aliphatic acyl group selected from acetyl, butyryl, caproyl, octanoyl, decanoyl, lauroyl, myristyl, palmitoyl and stearoyl, or an unsaturated aliphatic acyl group selected from palmitoleyl, oleyl, linoleyl, and ricinoleyl; and the term "*aryl*" refers to a mono- or poly-carbocyclic aryl group, most preferably phenyl, optionally substituted by C₁ - C₄ alkyl, halogen and/or hydroxy.

Where m in the above formula I is 0, X is preferably $C_1 - C_{24}$ alkyl, most preferably methyl. By a preferred embodiment, the above composition comprises 1,2-cyclic propanediol phosphate or 1,3-cyclic propanediol.

By an additional embodiment m in formula I is 0 and X is CH_2OH . In accordance with this embodiment, preferably the composition comprises 1,2-cyclic glycerophosphate or phenyl 1,2-cyclic glycerophosphate.

By yet another embodiment, where m in formula I is 0, X is CH_2acyl and the composition preferably comprises 3-acyl-1,2-cyclic glycerophosphate, most preferably cyclic oleyl lysophosphatidic acid.

Where Y in the above formula I is $-CH_2-$, preferably the composition comprises 1,3-cyclic propanediol phosphate or phenyl 1,3-cyclic propanediol phosphate.

Where Y in the above formula I is $-CH(OH)-$ the composition preferably comprises 1,3-cyclic glycerophosphate or phenyl 1,3-cyclic glycerophosphate.

Where Y in the above formula I is $-C(=O)-$ the composition preferably comprises cyclic dihydroxyacetone phosphate or phenyl cyclic dihydroxyacetone phosphate.

Where R in the above formula I is a cation, it is preferably Na.

The GCs of the invention may exert one of many neural promoting activities including but not limited to promotion of neuronal outgrowth, promotion of nerve growth, provision of dopaminotrophic supporting environment in a diseased portion of the brain, prevention of nerve degeneration and nerve rescue. All these activities fall within the scope of neural promoting activity.

Thus, the present invention also provides a pharmaceutical composition for promoting neural activity comprising a pharmaceutical acceptable carrier and, as an active material, a compound of the general formula I above.

The ability of the pharmaceutical compositions of the invention to promote neural cell differentiation and neuronal activity in one or more of the above ways renders them extremely useful for treatment of various disorders. Thus, the invention also provides a pharmaceutical composition comprising a
5 pharmaceutical acceptable carrier and, as an active material, a compound of the general Formula I above, for the prevention or treatment of disorders and diseases which can be prevented or treated by promoting neural cell differentiation and/or neural activity.

Such disorders may be mental disorders such as, for example,
10 schizophrenia or dementia or disorders resulting in learning disabilities.

In addition, the pharmaceutical compositions of the invention may also be used for the treatment of neurodegenerative conditions involving damage to dopaminergic neural cells. Examples of such conditions are Alzheimer's disease (AD) or Parkinson's disease (PD).

15 Additional neurodegenerative conditions which are within the scope of the present invention are such which result from exposure of an individual to harmful environmental factors such as hazardous chemicals, neurodegenerative conditions resulting from a mechanical injury (e.g. injury of the optical nerve resulting from contact of the eye with an abusive external factor), etc.

20 Furthermore, it is known that, following primary degeneration of nerves, additional nerves present in the vicinity of the degenerated nerves undergo secondary degeneration. Treatment of an individual suffering from a primary neurodegenerative condition may prevent or reduce the appearance of secondary degeneration in additional nerves present in the vicinity of the
25 degenerated nerves. Such treatment, termed "*nerve rescue*" is also within the scope of the present invention.

By yet another of its aspects, the present invention provides a method for inducing promotion of neural cell differentiation of target cells comprising

contacting said target cells for a suitable period of time with an effective amount of a compound of the general formula I above.

A suitable period of time is such a period which enables the compositions of the invention to exert their activity. This period of time may easily be determined by a person skilled in the art for each kind of composition and target cells using any of the methods described herewith. Typically, and in contrast to some known factors which effect neural cells such as NGF, the period of time required for the GCs of the invention to be in contact with the target cells in order to exert their effect is very short (several minutes).

10 In accordance with an additional aspect of the invention, a method is provided for promoting neural activity in an individual comprising administering to the individual in need an effective amount of a compound of the general Formula I above.

15 A method for the prevention or treatment of disorders and diseases which can be prevented or treated by promoting neural cell differentiation and/or neural activity is also provided comprising administering to a person in need a therapeutically effective amount of a compound of Formula I above.

20 The method of the invention may be used for the treatment of a variety of disorders and diseases in which the abovementioned effects are beneficial, i.e., in which the effect of the GCs ameliorates or reduces the undesired phenomena of the treated condition or disease. These conditions and disorders may be for example, but not limited to mental disorders such as schizophrenia or dementia, disorders leading to learning disabilities, neurodegenerative disorders such as Alzheimer or Parkinson disease and for prevention or
25 treatment of nerve rescue following nerve injury.

DETAILED DESCRIPTION OF THE INVENTION

Cyclic glycerophosphates can be formed by enzymatic degradation of phospholipids which in most cases yields five or six membered ring cyclic

phosphates. The present invention encompasses within its scope both such cyclic glycerophosphates formed by enzymatic degradation of phospholipids as well as synthetically formed ones. CGs having rings of less than five or more than six carbon atoms are also included within its scope.

5 The cyclic phosphates of the invention may generally be synthesized using any one of the methods known in the art for synthesis of phosphate esters. Specific methods which may typically be used for preparing the cyclic phosphates of the invention are described specifically below (see Examples).

10 Analogous of these cyclic glycerophosphates of the invention are also within the scope of the invention being typically deoxy analogs as well as phenyl esters of the 1,3 cyclic phosphates. These analogs may also be prepared by enzymatic methods or synthetically by any of the methods known in the art.

15 In addition to the active material, the pharmaceutical compositions may also contain a carrier selected from any one of the carriers known in the art. The nature of the carrier will depend on the intended form of administration and indication for which the composition is used. Examples of such carriers are saline, pills or capsules, liposome suspensions. The compositions may also comprise a number of additional ingredients such as diluents, lubricants, binders, preservatives, etc.

20 Where the compositions of the invention are administered *in vivo*, a preferred mode of their administration either i.v., topically or per os although at times it may be advantageous to use other administration modes as well.

25 Typically, the pharmaceutical compositions of the invention will comprise about 1 mg to about 10 mg of the active material per kg body weight of the treated individual.

While the compositions of the invention will typically contain a single cGP, it is possible at times to include in the composition or to co-administer two or more cGPs which may then act together in a synergistic or additive manner to prevent or treat the neurogenerative disorder.

The cyclic phosphates of the invention may be used in any of their isomer forms, (see for example, the four stereo isomers which constitute the synthetic 1,3 cGP depicted in Appendix A). For various purposes, one of the isomers may be preferred over the remaining ones.

5 According to the invention, the cPGs may be administered either in a single dose or may be given repetitively over a period of time.

The compositions of the invention may also be administered to the treated individual in combination with an additional treatment, e.g. wherein the treated condition is a neurodegenerative one, the compositions may be given together
10 with one of the currently available drugs or therapies used for treatment of neurodegenerative diseases such as dopamine receptor stimulants, L-dopa or together with a growth factor such as NGF. In such a combination treatment the cPGs may be administered simultaneously with or at different times than the administration of the additional treatment so as to yield a maximum preventive or
15 therapeutic effect.

The cPGs of the invention may also be administered to cells *in vitro*. Such administration may result in outgrowth of nerve cells which may then be used for transplantation into an individual in need. In addition, administration of the compositions to neural cells may result in enhanced secretion of various growth
20 factors by these cells which, in turn, could also be used for treatment of neurodegenerative conditions alone or in combination with the cPGs of the invention.

The effective amount of the GC to be used *in vitro* in accordance with the invention may vary in accordance with the nature of the CG as well as the target
25 cells and can easily be determined by a person versed in the art by using any of the above-mentioned methods or any of the methods known in the art. For *in vitro* induction, the typical range of concentration of CG needed to induce phosphorylation is between about 0.5 μ M to about 10 μ M.

EXAMPLES

The invention will now be illustrated by the following non-limiting examples with reference to the appended figures.

5 BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows photographs of PC12 cells following their incubation for 48 hours with growth medium containing linear α glycerophosphate as control (Fig. 1A), with nerve growth factor (NGF) at a concentration of 50 ng per/ml (Fig. 1B) and with the cyclic glycerophosphate 1,3 cGP at a concentration of 1 μ M (Fig. 1C). Neuronal outgrowth is clearly seen in Figs. 1B and 1C.

Fig. 2 shows photographs of PC12 cells grown in culture medium (control) (Fig. 2A), pulsed for three hours with linear α , and β cyclic glycerophosphates (Fig. 2B and 2C, respectively) with the cyclic glycerophosphates 1,3 cGP, phenyl-1,3 cGP, 1,2, cGP, 1,3, cPP, and phenyl-1,3 cPP (Fig. 2D - Fig. 2H respectively) or with NGF (Fig. 2I). Following incubation, the cells were washed and grown in growth medium and the photographs show the cells on day 7 after treatment with the various factors. Neural outgrowth is seen only in PC12 cells treated with the above cyclic glycerophosphates (Figs. D-H).

Fig. 3 are photographs showing PC12 cells treated for a period of 7 days control medium (Fig. 3A), α GP (Fig. 3B), β GP (Fig. 3C), the cyclic GPs: 1,3 cGP, phenyl-1,3 cGP, 1,2, cGP, 1,3, cPP, and phenyl-1,3 cPP (Fig. 2D - Fig. 2H respectively) at a concentration of 0.5 μ M and with NGF (Fig. 3I). No neural outgrowth was observed after incubation with either α or β GP (Fig. 3B and C respectively) while neural outgrowth is observed to different extents in cells incubated with the various cyclic GPs (Fig. 3D-H). Under these conditions, extensive neural outgrowth is seen in cells grown with NGF (Fig. 3I).

CHEMICAL SECTION

Synthesis of the cyclic phosphates

The cyclic phosphates of the invention are prepared by the reaction of a suitable dihydroxy compound wherein Y is - $(CH_2)_m$ - or - $C(=O)$ - and X is H or alkyl with phosphorus oxychloride ($POCl_3$) when R is H or with aryl, e.g. phenyl, phosphorodichloridate ($RO-P(=O)Cl_2$) when R is aryl.

When there is one or more hydroxy groups in the starting compound, namely Y is - $CH(OH)$ - and/or X is - CH_2OH -, these hydroxy groups have to be protected, e.g. by benzylation, and the benzyl group is then removed after cyclization by conventional catalytic hydrogenation in the presence of a suitable catalyst such as Pt or Pd.

The reaction is carried out in an anhydrous solvent, e.g. dioxane or methylene chloride, in the presence of equivalent amounts of a nucleophile such as pyridine or triethylamine. The end products, when R is not aryl, are usually obtained as salts.

The synthesis of a series of known and novel 5- and 6-membered ring cyclic phosphates is illustrated below.

Example 1: Synthesis of 1,3 cyclic glycerophosphate (1,3 cGP)

The procedure of Buchnea (Buchnea, 1973) was followed essentially as described. Briefly, 2-benzyloxy-1,3-propanediol (Aldrich) was reacted with an equimolar amount of phosphorus oxychloride (Aldrich) in methylene chloride. The resulting 2-benzyl-1,3 cGP was treated with hydrogen under the catalysis of Pd black in methanol to remove the benzyl residue. The 1,3 cGP, isolated as the Ba salt, was pure on paper chromatography (n-propanol: ammonia: water 6:3:1, $R_f=0.52$).

1,3 cGP was also produced by the cleavage of phosphatidyl glycerol (PG) with phospholipase C as described (Shinitzky et al., 1993). The product had a trace of approx. 10-20% α -GP as indicated by paper chromatography.

Example 2: Synthesis of 1,2 cyclic glycerophosphate (1,2 cGP)

This compound was prepared as described (Kugel, L. and Halmann, M., *J. Am. Chem. Soc.*, **89**:4125-4128 (1967). The disodium salt of β -glycerophosphate (Sigma) was first converted to the acid form and then cyclized with
5 dicyclohexylcarbodiimide (Aldrich). The product, isolated as the Ba salt, was pure on paper chromatography.

Example 3: Synthesis of phenyl 1,3 cyclic glycerophosphate (P-1,3 cGP)

The method described in Example 1 for 1,3 cGP was followed by
10 reacting 2-benzyloxy-1,3-propanediol with phenyl phosphorodichloridate (Aldrich). The intermediate benzylated product was pure on thin layer chromatography (ethyl acetate:hexane 3:2 R_f = 0.58), with a melting point of 136°C. It was further hydrogenated as in Example 1 to remove selectively the
15 benzyl residue. The obtained P-1,3 cGP, compound III, was pure on thin layer chromatography (as above) with R_f = 0.15 and melting point of 116°C.

Example 4: Synthesis of 1,3 cyclic propanediol phosphate (1,3 cPP)

1,3 cPP was prepared by reacting 1,3-propanediol (Aldrich) with an
equimolar amount of phosphorus oxychloride and then purified as described by
20 Buwalda et al., 1997. The product was isolated as the free acid (melting point: 99-100°C).

^{32}P labeled 1,3 cPP (1,3 cP ^{32}P) was prepared with $^{32}\text{POCl}_3$. The latter
was obtained by introducing a trace of $\text{H}_3^{32}\text{PO}_4$ (Amersham) into an excess of
POCl₃ in the cold (Neuhaus and Korkes, 1958). The reaction was then
25 proceeded on a microscale and 1,3 cP ^{32}P was isolated by co-crystallization with
unlabelled 1,3 cPP.

Example 5: Synthesis of 1,2 cyclic propanediol phosphate (1,2 cPP)

1,2 cPP was prepared by the same procedure as in Example 4 but using 1,2- propanediol (Aldrich). The compound was isolated as the Ba salt and was pure on paper chromatography (n-propanol:ammonia:water 6:3:1, $R_f=0.55$).

5

Example 6: Synthesis of phenyl 1,3 cyclic propanediol phosphate (P-1,3 cPP)

P-1,3 cPP was prepared by a method analogous to the procedure of
10 Example 4, by reaction of 1,3-propanediol with an equimolar amount of phenyl phosphorodichloridate in dry pyridine. The product was crystallized twice from ethyl acetate-hexane and had a melting point of 72°C.

Example 7: Synthesis of phenyl 1,2 cyclic glycerophosphate (P-1,2 cGP)

15 This novel compound was prepared as in Example 3 by reaction of 1-benzyloxy-2,3-propanediol with phenyl- PO_2Cl_2 , followed by removal of the benzyl residue by selective hydrogenation. Crystallization was achieved from ethanol-acetone and the product had a melting point of 95°C.

20 **Example 8: Synthesis of phenyl 1,2 cyclic propanediol phosphate (P-1,2 cPP)**

This novel compound was prepared as in Example 6 by reaction of 1,2-propanediol with an equimolar amount of phenyl- PO_2Cl_2 in dry pyridine.
25 Crystallization was achieved from ethyl acetate-hexane and the product had a melting point of 69°C.

Example 9: Synthesis of cyclic dihydroxyacetone phosphate (cdAP)

This novel compound was prepared by reaction of POCl_3 with
30 dihydroxyacetone.

1.8 g (0.01M dimer or 0.02M monomer) Dihydroxyacetone dimer MW-180 dissolved in 20 ml fresh distilled methylene chloride.

3.07 g = 1.87 ml (0.02M) Phosphoryl Chloride (MW-153.5, d-1.645) in 4 ml MeCl₂ was slowly added to the solution at RT. The solution was refluxed for 15 h (the solution was black). Methylene chloride was evaporated and 100 ml 90% acetone/water was added to the solution. The reaction mixture was refluxed for 18 h. The black solution was treated with active carbon at RT and filtered. From the resulting slightly yellow solution was evaporated acetone and water and the very nice crystalline residue was dissolved in 10 ml acetone. 0.01 M BaJ₂ in 80 ml acetone was added to the solution and nice crystals of cyclic-dihydroxyacetone-phosphate barium salt started to precipitate. The precipitate was washed 3 times with small quantities of acetone and dried. The product was cleaned by dissolving it in small amounts of water and precipitating with acetone. The resulting produce is white crystalline powder and shows in paper chromatography (solvents mixture: n-Propanol:NH₄H₂O 6:3:1) R_f - 0.50.

Example 10: Synthesis of phenyl cyclic dihydroxyacetone phosphate (P-dAP)

This novel compound was prepared by reaction of phenyl-PO₂Cl₂ with dihydroxyacetone in dry pyridine. Upon removal of the solvent by vacuum, the residue was extracted twice with ethyl acetate. After evaporation of the ethyl acetate, an oily residue was obtained..

Example 11: Synthesis of cyclic oleyl lysophosphatidic acids (c-oLP)

These novel compounds were prepared by reaction of oleyl lysophosphatidic acid (Avanti Polar Lipids) with excess dicyclohexylcarbodiimide (DCC) in dimethyl sulfoxide. The product appeared as a oil.

BIOLOGICAL SECTION

Materials and Methods

PC12 cells originated from rat pheochromaytoma and were grown as monolayers in Eagle's medium (EM) supplemented with 10% fetal calf serum, 50 µg/ml gentamicin and 5 mM glutamine, in a humidified incubator buffered with 5% CO₂, at 37°C. The culture medium is changed every four days and the cells are passaged every eight days and performed confluent monolayers (1.5 x 10⁶ in a 10 cm plate or 10⁵ cells per well in 24 wells plate). PC12 cells are originally round cells which, following several days in the presence of nerve growth factor (NGF) process nerves. Upon withdrawal of the NGF, the nerves retract and a process of apoptosis is initiated in the cells.

Induction of PD in rats

Sprague-Dawley (SD) rats (weighing 230-250 g) are anesthetized with ketamine plus xylazine administered i.p. and their head secured in a stereotaxic frame. 6OH-DA (8 mg/4ml) is then injected into the median-forebrain-bundle to destroy the dopaminergic terminals unilaterally (Fitoussi, N., *et al. Neuroscience*, **85**(2):405-413) (1998)). Manifestations of the disease are evident within 2-3 weeks.

Dopaminergic ablation is assessed behaviorally using a rotometer test, which is based on upregulation of dopamine receptors on the lesioned side. Systemic administration of a DA agonist (apomorphine, 0.25 mg/kg s.c.) induces rotation in rats with unilateral dopaminergic ablation, with rotation occurring in the direction contralateral to the side of the lesion.

25

Administration of cyclic glycerophosphates into the brain

Cyclic phosphates are administered into the brain using ALZET osmotic pumps (ALZET Corporation, Palo Alto, CA). A canulla (30 gauge) is implanted 0.5 mm medial to the SN of rats, using a stereotaxic device after

assessment of nigrostriatal lesions (rotation behavior). Cyclic phosphates are microperfused at a rate of 1 μ l/h for 3 or 14 days.

Brain dissection and extraction

5 Rats are decapitated and their brains rapidly removed. The brains are then placed in a rat brain mold on ice and 0.5 mm serial sections are cut and placed on chilled microscope slides. Tissue punches are rapidly taken using a stainless steel cannula with an inner diameter of 1.1 mm, according to the following coordinates: A1.5-2.0 mm for the striatum; P5.5-5.0 mm for the SN,
10 and include most of the desired regions. The tissue samples are immediately frozen in liquid nitrogen and stored at -70°C until extraction. Extraction is achieved by thawing the punches and subjecting them to probe sonication (80 watts for 5 sec. with a Sonifier B-12; Branson, Danbury, CN) in 0.5 ml of a perchlorate solution (0.1M) containing EDTA/ethanol (0.021%) on ice. A
15 sample (100 μ l) is removed for protein analysis and the remainder is centrifuged (2000 x g, 10 mins. 4°C). The resulting supernatants (the tissue extracts) are filtered (0.45 μ m Acrodisk, Gelman; Ann. Arbor. MI) and stored at -70°C until subjected to ELISA analysis to determine IIs or GDNF or HPLC analysis to determine the 5-HT and 5-HIAA content

20

Assessment of GDNF

The effect of cyclic glycerophosphates on the release and production of GDNF from the SVG-cells and brain tissue extracts is determined as follows.

Cells are incubated for 12, 24 and 48 hours with or without cyclic phosphates.

25 Supernatants are taken after centrifugation and analyzed for GDNF using an ELISA kit (ENDOGEN, MA, USA and PROMEGA, Madison, USA, respectively).

Isolation of RNA

Total RNA is isolated from cultured cells or tissue extracts, using Tri Reagent™ (Boehringer Mannheim, Germany). Cells are lysed in the reagent (10⁶ cells/1 ml reagent). Frozen tissue punches are homogenized with the reagent (50 mg tissue/1 ml) using a glass Teflon rod. Chloroform is then added and the homogenates are separated into three phases by centrifugation. Care is taken when removing the aqueous phase so as not to disturb the interphase or the organic phase. In order to avoid genomic DNA contamination, RNA is precipitated from the aqueous phase by addition of isopropanol, washed with ethanol and solubilized in DEPC treated water. RNA is estimated spectrophotometrically at 260 nm and 280 nm and stored at -80°C until use.

First strand cDNA synthesis is carried out in a reaction volume of 20 µl containing 3 µg of total RNA, 10 mM primer dT (Boehringer Mannheim, Germany) and 1 mM dNTP mix (Boehringer Mannheim, Germany). After heating for 2 min. at 65°C and cooling back to 4°C, the reaction is initiated by the addition of 50 units M-MuLV reverse transcriptase and 20 units RNase inhibitor (Boehringer Mannheim, Germany). The mixture is then brought to 37°C for 60 mins. PCR on the cDNA was carried out in a reaction volume of 50 µl. First strand cDNA (2 µl) is added to the PCR mixture containing the following components: 0.2 mM dNTP mix (Boehringer Mannheim, Germany), 1 mM each oligonucleotide primer (primers were designed according to the published GDNF cDNA sequence. 5'-TCACCAGATAAACAATGGC-3' {5'} and 5'-TACATCCACACCTTTTAGCG -3' {3'} corresponding to bases 81-101 and 460-480 respectively) (Biosource, CA, USA), and 2.5 U Taq DNA polymerase (Boehringer Mannheim). Reactions are overlaid with mineral oil, and initially denatured at 94°C for 2 min. PCR is performed using a MJ Research thermal cycler programmed for 40 cycles consisting of denaturation at 94°C for 1 min. followed by primer annealing at 55°C for 1 min. and primer extension at 72°C for 1 min. At the end of the 40 cycles, the reaction mixture is

kept at 72°C for 10 min. The PCR product is electrophoretically analyzed on a 2% agarose gel containing ethidium bromide).

Immunohistochemical assessment of the cell survival in the brain

5 At the end of the experiment the animals are anesthetized with ketamine and xylazine i.p. and then perfused via cardiac puncture with PBS followed by 4% paraformaldehyde. The brains are then removed and post-fixed in 4% paraformaldehyde for 24 hrs and then transferred into 20% sucrose for 48 hours. Tissue sections of 35 μ m are obtained using a cryostat and placed in 24
10 wells plate in PBS. The sections are incubated overnight in 4°C with a primary rabbit polyclonal antibody to Tyrosine hydroxylase (TH) (Chemicon, CA, USA) or a primary mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) (Chemicon, CA, USA). The sections are then washed with PBS, incubated (1 hr) with a HRP conjugated secondary antibody (sheep anti-rabbit
15 or anti-mouse) (Chemicon, CA, USA) and washed with PBS. Then, the sections are incubated with the chromagen diaminobenzidine (DAB), counter-stained with hematoxylin, and screened by light microscopy. Positive staining for TH indicates the amount of dopaminergic- cells in the striatum and substantia nigra, i.e. dopaminergic- cells survival. Positive staining for the GFAP in the injection
20 tract indicates glioma processes.

Microdialysis

The microdialysis technique requires implantation of a small (500 μ m diameter) probe into the brain of live rats. Implanation is performed on rats as
25 follows. A rat is anesthetized with pentobarbital, placed in a stereotaxic apparatus, a burr hole drilled through its skull according to stereotaxic coordinates, and a commercially available microdialysis probe (CMA/10 probe of 3 mm length, 20 kD cutoff values; Carnegie Medicine, West lafayette, IN) will be lowered into the striatum. Artificial cerebrospinal fluid (CSF; 145 mM

NaCl, 1.2 mM CaCl₂, 2.7 mM KCl, 1.0 mM MgCl₂, pH 7.4) is perfused slowly (1 µl/min) through the probe. Small molecules will diffuse between the artificial CSF and the extracellular fluid of the brain tissue. The rats are allowed to recover for 20-24 hrs, after which dialysate is collected from the effluent of the microdialysis probe. Dialysates (30 µl during 30 mins. intervals) are collected in polyethylene tubes containing 15 µl of EDTA-ethanol (0.02/1%) as a preservative at baseline and during administration of DA antagonists via the probe. The collected dialysates are stored at -70°C until subjected to HPLC analysis.

Analysis of monoamines and metabolites in tissue extracts and microdialysates

The frozen tissue extracts and microdialysates are thawed, and injected directly into a HPLC apparatus (Altex Ion Pair Ultrasphere C18, 4.6 mm inside diameter 250 mm Column No. 235335) coupled to an electrochemical detector (Model 460; Waters; Milford, MA) with an oxidation potential of 0.70 to 0.78 V. the mobile phase consists of 2 liters of water, 0.55 g of 1-heptanesulfonic acid, 0.2 g of EDTA, 16 ml of triethylamine, 12 ml of 85% phosphoric acid, and 80 ml of acetonitrile, pumped at 0.8 ml/min. In each sample subjected to HPLC, the levels of DA, as well as its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are determined by this procedure.

RESULTS

Example 12

PC12 cells were grown in culture as explained above, the cells were divided into three groups and different factors were added to the growth medium of each group for 48 hours as follows:

Group A - α glycerophosphate.

Group B - nerve growth factor (NGF) at a concentration of 50 ng/ml.

Group C - 1,3 cGP at a concentration of 1 μ M.

5 The rate of neuronal growth in each of the above cultures was monitored and documented by microscopic photographs. As seen in Fig. 1, while growth of the cells in the presence of α glycerophosphate did not promote neural outgrowth in the cells (Fig. 1A) such neural outgrowth was clearly seen in the cells which were grown in the presence of NGF (Fig. 1B) or 1,3 cGP (Fig. 1C).

Example 13

10 Cells were grown as described in Example 12 above with the same factors and at distinct stages the level of intercellular signaling proteins were evaluated by a Western Blot technique using antibodies specific for the tested proteins.

Example 14

15 Cells were grown as described above and divided into groups which were each grown with one of the following:

(A)	growth medium	(B)	α GP	(C)	β GP
(D)	1,3 CGP	(E)	phenyl 1,3 cGP	(F)	1,2 cGP
20 (G)	1,3 cPP	(H)	phenyl-1,3 cPP	(I)	NGF

The above factors were added to the cells for a period of three hours after which they were washed away from the cells. The cells were further grown in a growth medium which did not comprise the above factors. The neural outgrowth
25 of the cells was monitored and microscopic photographs were taken on day seven after treatment with the above factors.

As seen in Fig. 2, under the above conditions, neural outgrowth was seen only in the cells incubated with the above cGPs (Fig. 2D-2H). No neural outgrowth was seen in the cells incubated with the linear glycerophosphates (Fig.

2B and C) and under the above conditions NGF did not promote any nerve generation as well (Fig. 2I).

Example 15

5 PC12 cells were grown as described above and divided into the same groups as described in Example 14 above. The cells were incubated with the various factors for a consecutive period of seven days. Neural outgrowth was monitored and microscopic photographs were taken following the seven day incubation with the above factors.

10 As seen in Fig. 3, neural outgrowth was seen in cells incubated with the various above cGPs (Fig. 3B-3H) as well as in cells grown in the presence of NGF (Fig. 3I) but not in the control cells grown with the linear glycerophosphates (Fig. 3A).

15 Example 16

PC12 cells are grown in culture in the presence of NGF under conditions allowing neuronal outgrowth of the cells. The NGF is then withdrawn by washing the cells and replacing their growth medium with a medium comprising no NGF. The cells then retract and the nerves disintegrate (analogous to the delayed neurodegeneration observed in the vicinity of injured nerves). Following this integration, the tested cGP is added to the culture either for a short period of time after which it is washed or for a longer period of time and the cPEs capability of "rescuing" the nerves is assessed by evaluating the re-differentiation of the cells into neuronal cells.

25

Example 17

Parkinson's disease is induced in rats as described in the Materials and Methods part above by injection of 6OH-DA into their brains.

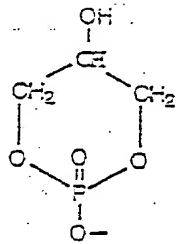
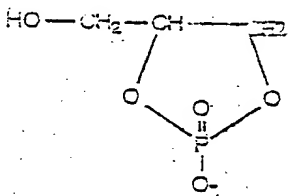
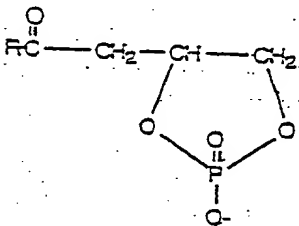
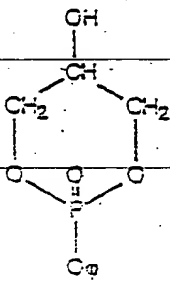
The rats are then treated either with α and β linear GPs or with cGP by administration of the glycerophosphates either topically, per os, or directly into the brain using osmotic pumps as described above.

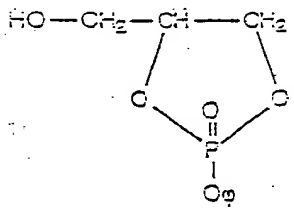
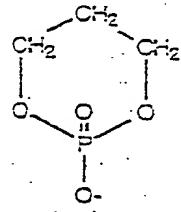
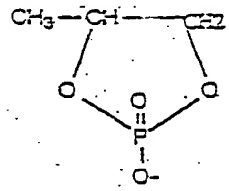
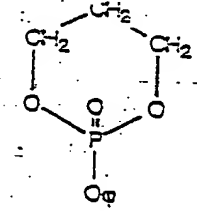
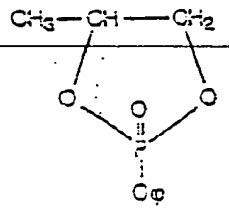
5 The effect of the linear and cyclic GPs is assessed by evaluating the *in situ* production of L-DOPA, dopamine (DA), the dopamine metabolites DOPAC and HVA and the growth factor GDNF by using microdialysis techniques and by the methods described above. Motional and limb tremor parameters are also quantitatively evaluated in the rats treated with each of the above factors.

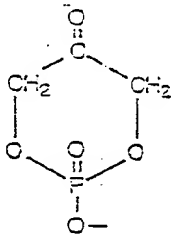
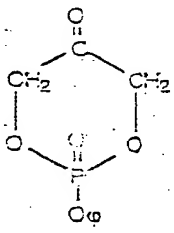
10 Example 18

Rats having injured optical nerves are treated with α and β linear glycerophosphates or with a cyclic glycerophosphate as explained above and the effect of the above glycerophosphates on the visual response and nerve generation of the treated rats is monitored.

Appendix A

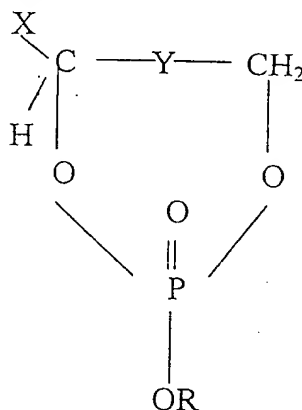
	Formula	Abbreviation
I		1,3 cGP
II		1,2 cGP
III		cyclic lysophosphatidic acid, c-lysoPA
IV		P-1,3 cGP

	Formula	Abbreviation
V		P-1,2 cGP
VI		1,3 cPP
VII		1,2 cPP
VIII		P-1,3 cPP
IX		P-1,2, cPP

	Formula	Abbreviation
X		cDHAP
XI		P-cDHAP

CLAIMS:

1. A pharmaceutical composition for promoting neural cell differentiation in target cells comprising a pharmaceutically acceptable carrier and, as an active material, a compound of the general formula I



wherein

Y is $-(\text{CH}_2)_m-$, $-\text{CH}(\text{OH})-$ or $-\text{C}(=\text{O})-$, and m is 0 - 3 ;

X is H, alkyl, $-\text{CH}_2\text{OH}-$, CH_2Oacyl or $-\text{CH}_2\text{acyl}$; and

R is H, a cation, alkyl or optionally substituted aryl;

or an analog of said compound.

2. A pharmaceutical composition for promoting neural activity comprising a pharmaceutical acceptable carrier and, as an active material, a compound of the general Formula I of Claim 1.

3. A pharmaceutical composition according to Claim 2, wherein said neural activity is selected from the group consisting of promotion of neuronal outgrowth, promotion of nerve growth, provision of dopaminotrophic supporting environment in a diseased portion of the brain, prevention of nerve degeneration and nerve rescue.

4. A pharmaceutical composition comprising a pharmaceutical acceptable carrier and, as an active material, a compound of the general Formula I of Claim 1, for the prevention or treatment of disorders and diseases which can be

prevented or treated by promoting neural cell differentiation and/or neural activity.

5. A pharmaceutical composition according to Claim 4, wherein said disorders and diseases are mental disorders.

5 6. A pharmaceutical composition according to Claim 5, wherein said mental disorder is schizophrenia or dementia.

7. A pharmaceutical composition according to Claim 5, wherein said mental disorder is a learning disability.

8. A pharmaceutical composition according to Claim 4, for the treatment of
10 neurodegenerative conditions involving damage to the dopaminergic neural cells.

9. A pharmaceutical composition according to Claim 8, wherein said neurodegenerative condition is Alzheimer's disease.

10. A pharmaceutical composition according to Claim 8, wherein said
15 neurodegenerative condition is Parkinson's disease.

11. A pharmaceutical composition according to Claim 4, wherein said disorders and diseases result from exposure to harmful environmental factors or from a mechanical injury.

12. A pharmaceutical composition according to Claim 4, for the treatment of
20 nerve rescue after nerve injury.

13. A method for inducing promotion of neural cell differentiation of target cells comprising contacting said target cells for a suitable period of time with an effective amount of a compound of the general Formula I of Claim 1.

14. A method for promoting neural activity in an individual comprising
25 administering to the individual in need an effective amount of a compound of the general Formula I of Claim 1.

15. A method according to Claim 14, wherein said neural activity is selected from the group consisting of promotion of neuronal outgrowth, promotion of nerve growth, provision of dopaminotrophic supporting environment in a

diseased portion of the brain, prevention of nerve degeneration and nerve rescue.

5 16. A method for the prevention or treatment of disorders and diseases which can be prevented or treated by promoting neural cell differentiation and/or neural activity comprising administering to a person in need a therapeutically effective amount of a compound of Formula I of Claim 1.

17. A method according to Claim 16, wherein said disorders and diseases are mental disorders or diseases.

10 18. A method according to Claim 17, wherein said mental disorder or disease is schizophrenia or dementia.

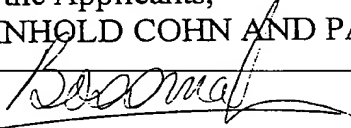
19. A method according to Claim 16, wherein said mental disorder is a learning disability.

20. A method according to Claim 16, wherein said disorders and diseases are neurodegenerative disorders or diseases.

15 21. A method according to Claim 20, wherein said neurodegenerative disorder or disease is Alzheimer's disease or Parkinson's disease.

22. A method according to Claim 16, wherein said disorders or diseases result from exposure of an individual to harmful environmental factors or from a mechanical injury.

20 23. A method according to Claim 14, for the treatment of nerve rescue after nerve injury.

For the Applicants,
REINHOLD COHN AND PARTNERS
By: 

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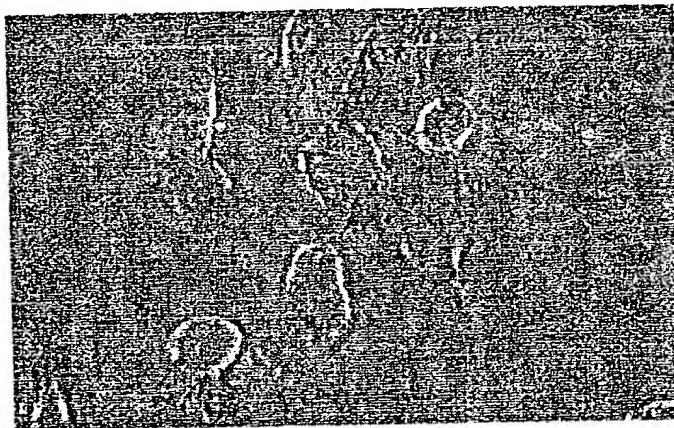


FIG. 1A

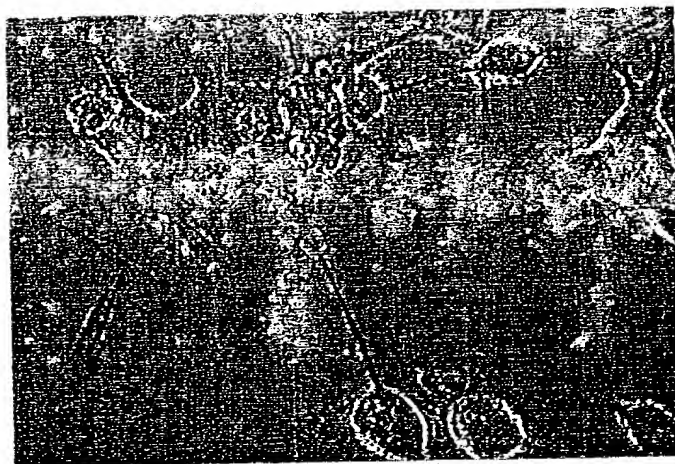


FIG. 1B



FIG. 1C

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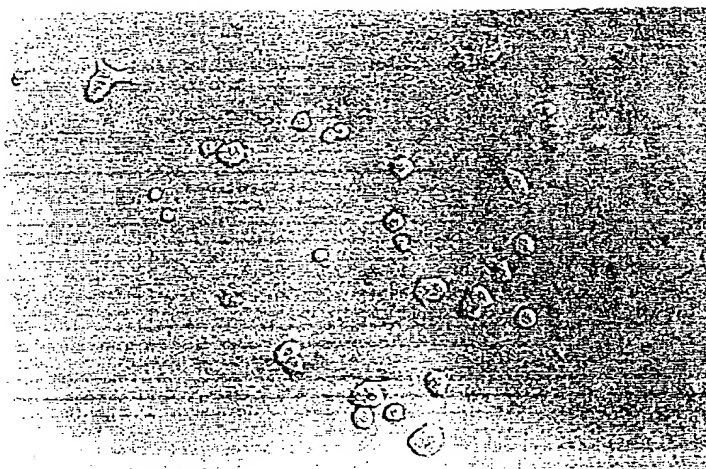


FIG. 2A

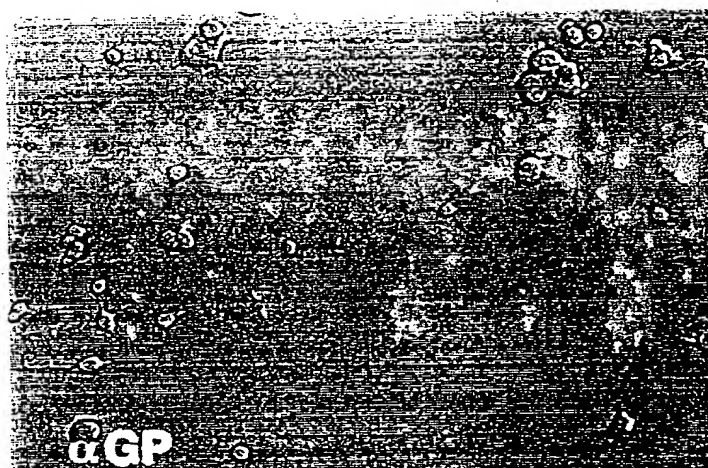


FIG. 2B

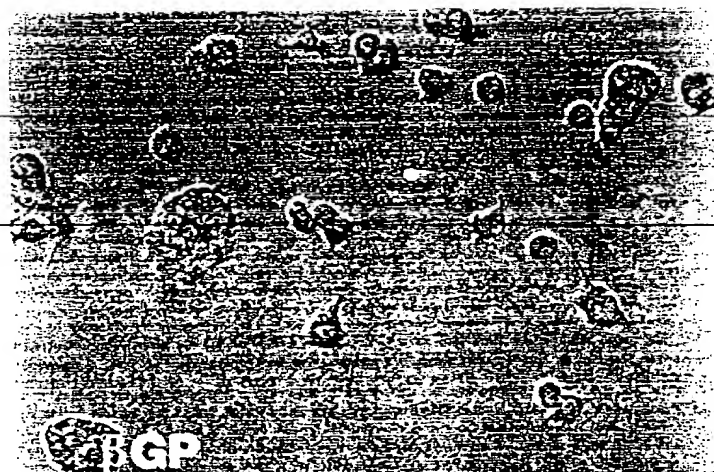


FIG. 2C

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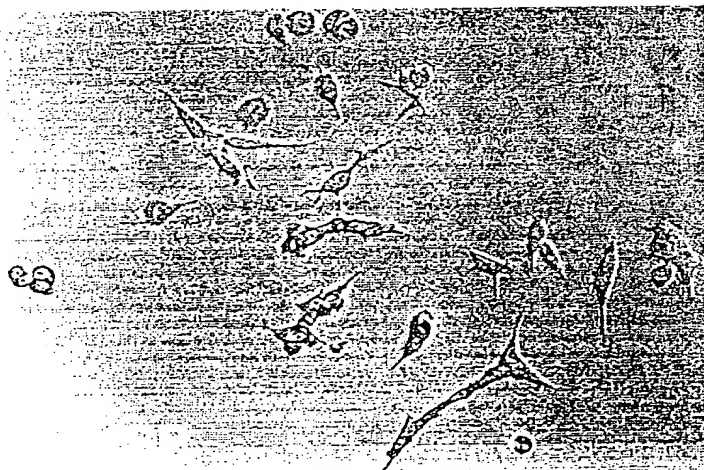


FIG. 2D



FIG. 2E

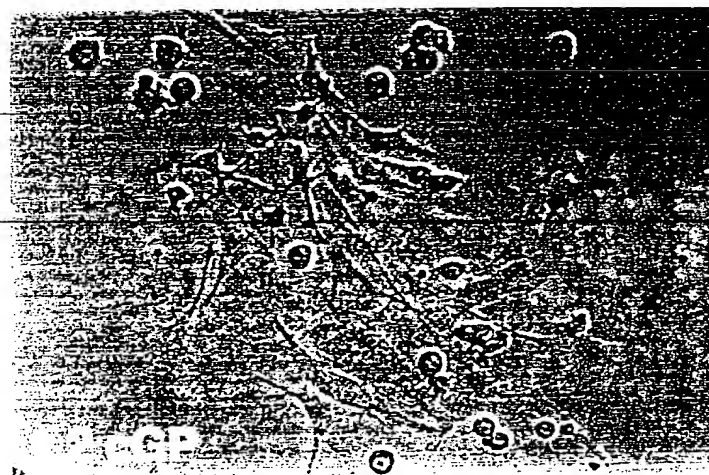


FIG. 2F

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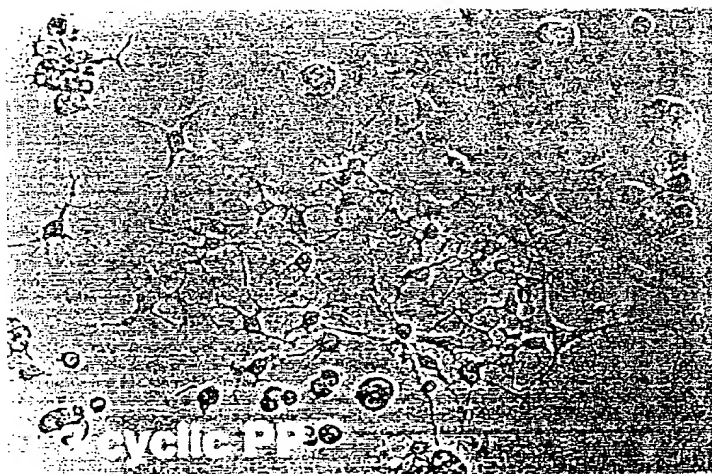


FIG.2G

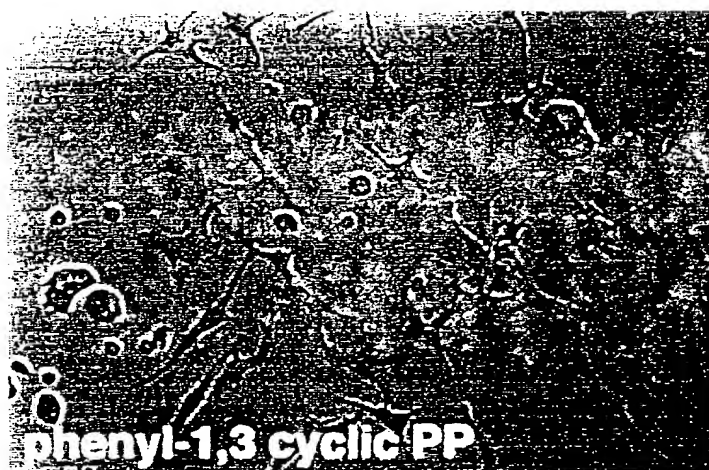


FIG.2H

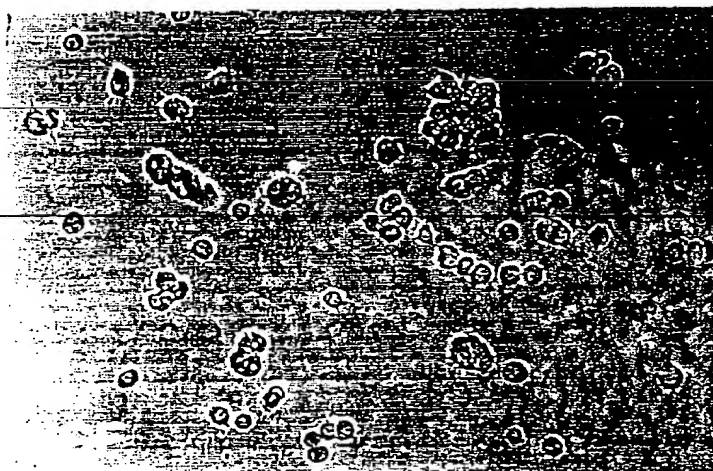


FIG.2I

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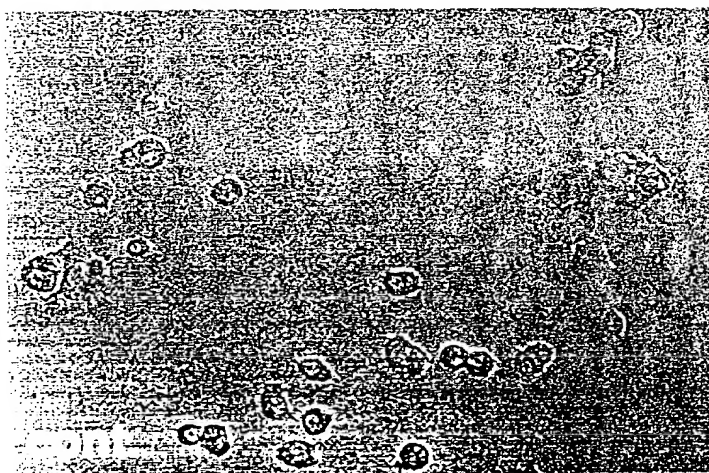


FIG. 3A

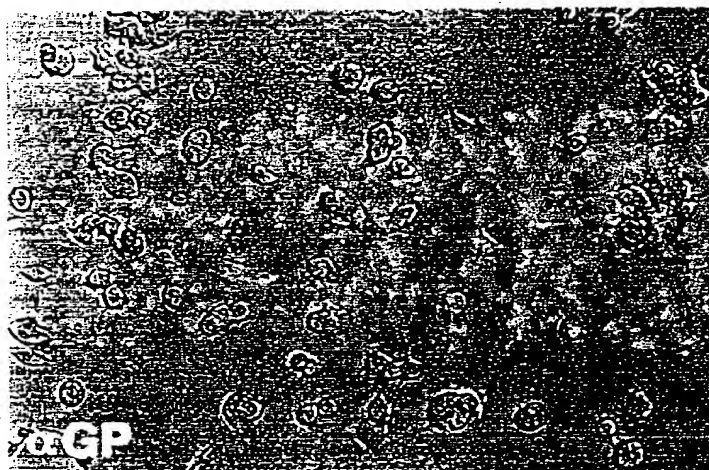


FIG. 3B

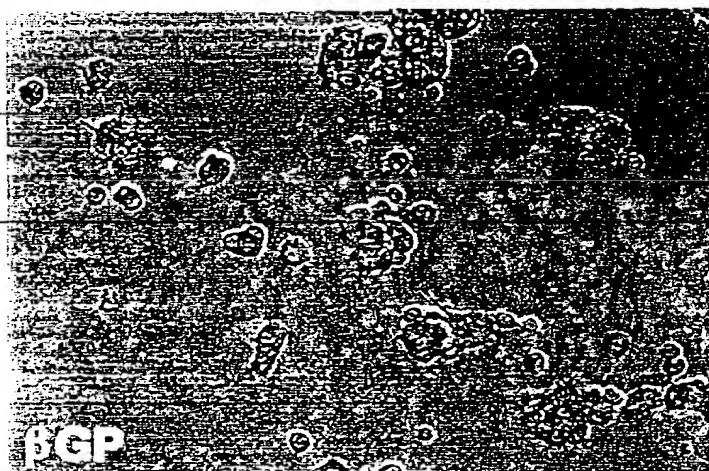


FIG. 3C

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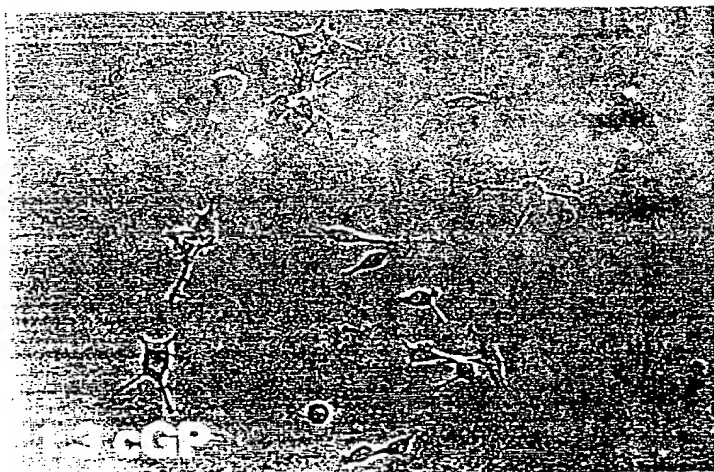


FIG.3D

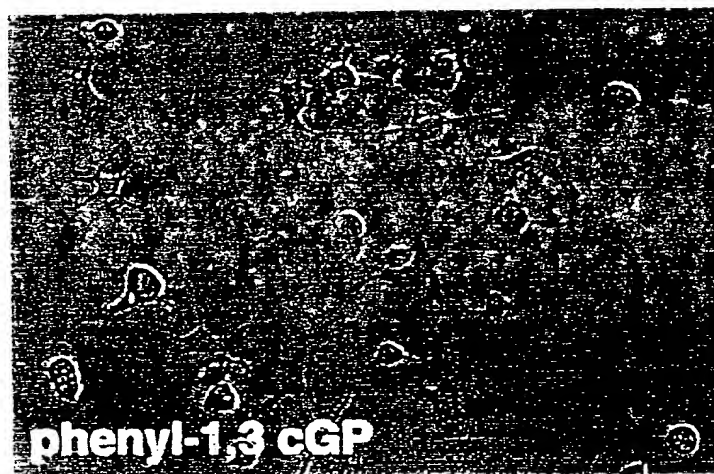


FIG.3E

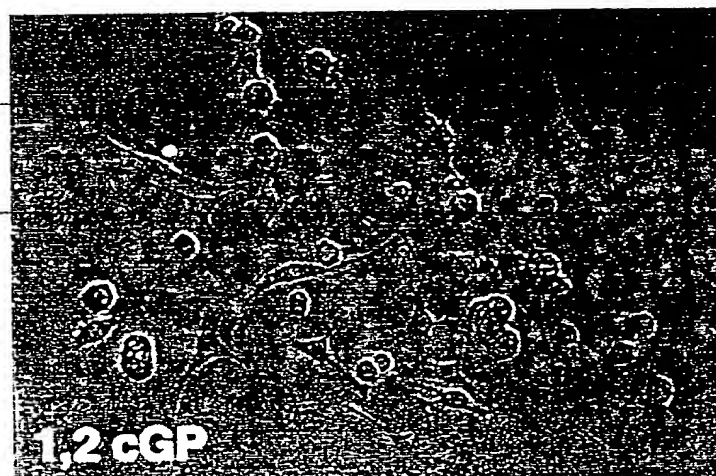


FIG.3F

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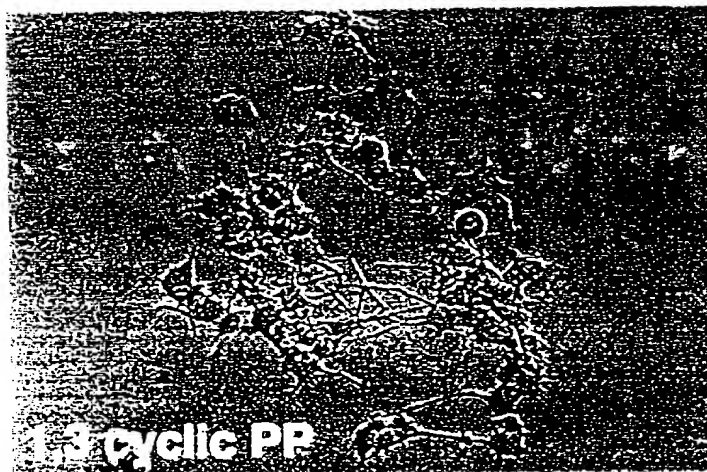


FIG.3G

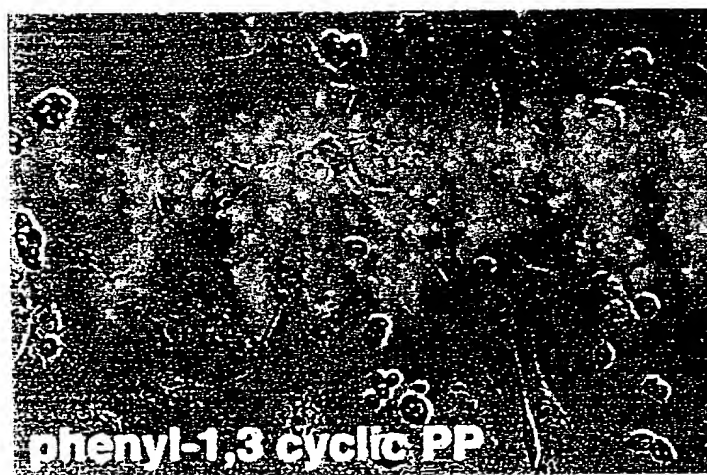


FIG.3H

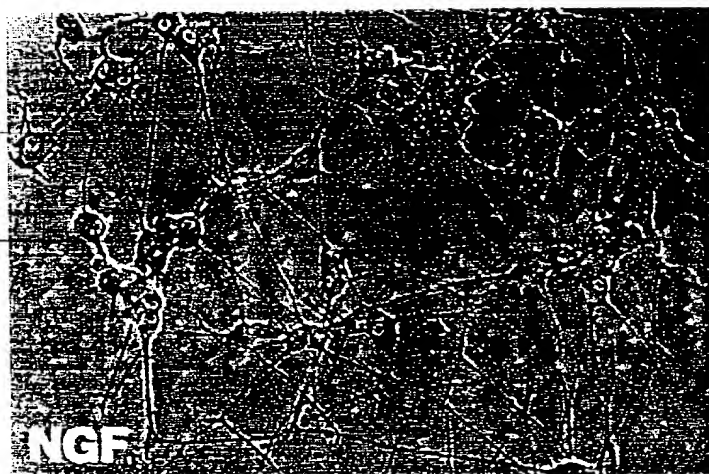


FIG.3I

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